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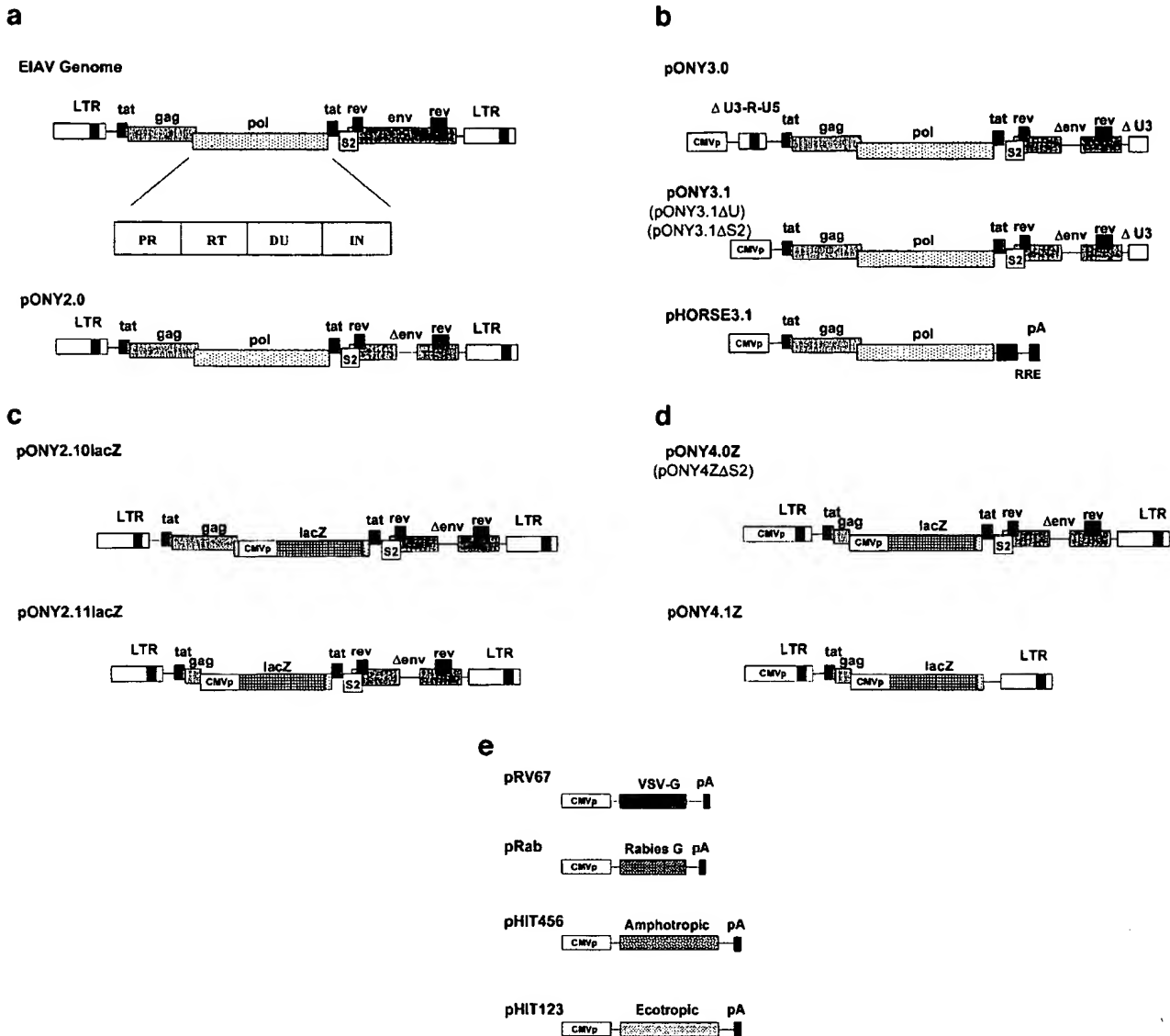
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**Figure 1** Plasmids used in this study. (a) The genomic organization of EIAV and the starting plasmid pONY2.0 is shown. The location of all of the genes and the LTR are indicated and the position of the dUTPase within the pol gene is shown (DU). PONY2.0 contains a 0.7 kb deletion in the env gene. (b) Schematic diagrams of the gag/pol expression plasmids. Designations in brackets indicate derivatives of the plasmids that have mutations as discussed in the text. (c) Schematic diagrams of the vector genome plasmids are shown. Designations in brackets indicate derivatives of the genome that have mutations as discussed in the text. CMVp refers to the human cytomegalovirus immediate-early enhancer/promoter. (d) Schematic diagrams of the envelope expression plasmids are shown. pA is the SV40 large T polyadenylation signal.

unknown.<sup>16</sup> The non-primate lentiviruses also contain an extra domain within the pol gene that encodes a dUTPase.<sup>17</sup> This has been implicated in the ability of the non-primate lentiviruses to replicate in mitotically inactive cells such as macrophages.<sup>18–23</sup>

We have made a replication defective EIAV vector based on a non-pathogenic infectious proviral clone pSPEIAV19 (accession number: U01866), a kind gift from SL Payne.<sup>24</sup> This vector system formed transducing viral particles with properties that led us to conclude that EIAV-based vectors provide a clear alternative to vectors based on the primate immunodeficiency viruses. They provide a means, therefore, of working with a powerful lentiviral vector system, in the clinic and in the labora-

tory, without the real or perceived hazards of dealing with a derivative of the causative agent of AIDS.

## Results

### EIAV-based constructs

Viral vector particles based on EIAV were generated by a three plasmid co-transfection system in which plasmids expressing gag/pol, envelope and genome were used as a mixed DNA preparation.<sup>25</sup> The EIAV envelope was not used in any of the experiments, instead the particles were pseudotyped with various non-EIAV proteins. This substantially reduces the probability of the production of replication competent EIAV.



## Stable gene transfer to the nervous system using a non-primate lentiviral vector

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We have constructed a non-primate lentiviral vector system based on the equine infectious anaemia virus (EIAV). This system is able to transduce both dividing and non-dividing cells, including primary cultured hippocampal neurons and neurons and glia in the adult rat central nervous system (CNS), at efficiencies comparable with HIV-based vectors. We demonstrate that the only EIAV proteins required for this activity are gag/pol and that the only accessory protein

required for vector production is rev. In addition, we show that the pol encoded dUTPase activity that is found in all non-primate lentiviruses is not required. The vectors can be pseudotyped with a range of envelopes including rabies G and MLV 4070A and can be concentrated to high titres. The ability of EIAV to infect mitotically inactive cells makes this vector an attractive alternative to the immunodeficiency viruses for gene therapy.

**Keywords:** lentiviral/retroviral vectors; rabies-G; dUTPase; S2; EIAV; CNS

### Introduction

Retrovirus-based vectors have been used extensively for gene delivery in the laboratory and in a variety of gene therapy trials. Their main advantages are ease of manipulation, predictable integration and reliable gene expression and regulation. The most significant disadvantage of these oncoretrovirus vectors has been their inability to transduce non-dividing cells or cells that are dividing slowly. This property has precluded their use *in vitro* and *in vivo* in a number of situations where terminally differentiated cells are the targets or where the physiological conditions of a target cell population leads to low mitotic indices. The need for gene transfer technology with the advantages of the oncoretrovirus vectors but without the disadvantage of being limited to dividing cells has led to the development of vectors based on the lentiviruses.

Lentiviruses are characterised by their slow, non-oncogenic pathogenesis and by their complex genomic organisation. From sequence similarity studies, the lentiviruses can be subdivided into the primate and non-primate viruses.<sup>1</sup> The primate viruses comprise the human and simian immunodeficiency viruses (HIV and SIV) and the non-primate viruses include feline and bovine immunodeficiency viruses (FIV and BIV), caprine arthritis/encephalitis virus, visna/maedi/ovine progressive pneumonia virus, and equine infectious anaemia virus (EIAV). A major difference between the oncoretroviruses such as murine leukaemia virus (MLV) and the lentiviruses is that lentiviruses are capable of replicating in both dividing and non-dividing cell populations.<sup>2</sup>

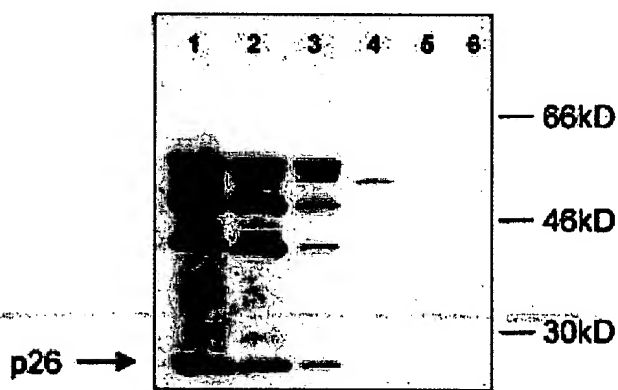
Most lentiviral vector development has focused on HIV-1-derived systems as HIV is the most thoroughly characterised of the lentiviruses.<sup>3,4</sup> These vectors have been shown to transduce a number of non-dividing cells *in vitro* and *in vivo* and the most advanced vectors have been minimised by removing all of the regulatory (except for rev) and so-called accessory genes, tat, nef, vpu, vpr, vif.<sup>5,6</sup> These HIV-based vectors are therefore highly disabled and replication competent virus cannot be detected in these systems. However, despite these disabling changes to the HIV-based vectors it is possible that, for diseases other than HIV infection, they may not be acceptable for gene therapy. These concerns are leading to a move away from HIV as a generally useful lentiviral vector to the use of non-primate viruses.<sup>7,8</sup> To date, vectors based on two non-primate retroviruses have been published. In both cases the results are preliminary in that the efficacy of these vectors has not been tested *in vivo*. Here we describe such a non-primate lentiviral vector system based on EIAV and demonstrate stable and long-term transduction of neural cells both *in vitro* and *in vivo*.

Equine infectious anaemia virus infects all equidae resulting in plasma viremia and thrombocytopenia.<sup>9</sup> Virus replication is controlled by the maturation of monocytes into macrophages. The latter is thought to be the sole cell type in which virus replicates *in vivo*.<sup>10</sup> It is not clear where this tropism restriction resides. The virus does not replicate in human cells.<sup>11</sup>

EIAV has one of the simplest genomic structures<sup>1</sup> of all the lentiviruses (Figure 1a). In addition to the gag, pol and env genes EIAV encodes three other genes: tat, rev and S2. Tat acts as a transcriptional activator of the viral LTR via its target RNA sequence TAR<sup>12,13</sup> and rev regulates and co-ordinates the expression of viral genes through rev-response elements (RREs) in unspliced or partially spliced transcripts.<sup>14,15</sup> The function of S2 is

To produce the EIAV structural and regulatory proteins a derivative of the EIAV genome containing a 0.7 kb deletion in the *env* gene (pONY2.0) was inserted into an expression plasmid (pCI-Neo; Promega, Madison, WI, USA) to produce pONY3.0 (Figure 1b). This contains a U3 deletion in the 5' LTR and lacks R and U5 in the 3' LTR and so cannot complete reverse transcription. To minimize the potential for interference from transcription from the upstream LTR and to increase protein expression the remaining portions of the 5' LTR from pONY3.0 were deleted creating construct pONY3.1 (Figure 1b). Both pONY3.0 and pONY3.1 express gag/pol, tat, rev and S2. pONY2.0 expresses the gag/pol proteins from the EIAV LTR in response to tat while pONY3.0 and pONY3.1 are tat independent and express the proteins from the human cytomegalovirus immediate-early enhancer/promoter (CMV). Although pONY3.0 contains a functional U3 the majority of the transcripts found to initiate from the CMV promoter were based on primer extension analysis (data not shown). All of the expression plasmids contain the SV40 origin of replication and 293T cells were used for viral vector production.

The initial vector genome pONY2.10lacZ (Figure 1c) was derived from pONY2.0 by a small deletion of gag and most of the pol gene (see Materials and methods). This can express tat, rev, S2 and truncated gag/pol proteins from the EIAV LTR expression signals. The presence of gag proteins in particle preparations derived from cells containing the gag/pol expression plasmids or the vector genome in pONY2.10lacZ were assessed by Western blotting (Figure 2 lanes 1 to 4; pONY2.11 shown in this Figure is discussed later). As expected, the vector genome produced a truncated gag protein (lane 4) but no processed products because of the lack of a functional pol gene. The gag/pol expression plasmids all produced gag



**Figure 2** Western blot analysis of viral proteins in cell supernatants. 20 µg of plasmids pONY3.1, pONY3.0, pONY2.0, pONY2.10lacZ, pONY2.11 and pCI-Neo (lanes 1, 2, 3, 4, 5, 6 respectively) were transfected into 293T cells. Forty-eight hours after transfection the cells supernatant was collected and pelleted by centrifugation for 30 min at 16000 g at 4°C. The equivalent of 1 ml of viral supernatant was loaded on the gel. The proteins were separated on 10% SDS-polyacrylamide gel. Expression of viral proteins was visualized using EIAV positive horse serum that was a kind gift from RC Montelaro (Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine). Molecular weight markers are indicated (Rainbow high molecular weight range; GIBCO BRL, Paisley, UK) and the major core protein, p26 is indicated. Higher molecular weight bands are consistent in size with the expected p55 precursor and processing products.

proteins including precursor and cleavage products with significant amounts of the p26 capsid protein that is predominantly recognised by the polyclonal horse serum used.<sup>26</sup> These data therefore indicate the presence of an active protease and authentic gag expression. Expression of gag/pol was somewhat lower from pONY2.0 (lane 3) indicating that the EIAV LTR in the presence of tat was less effective than the CMV promoter.

#### Pseudotyping of EIAV vectors

Having constructed a simple vector genome and established that the gag/pol cassettes were indeed producing gag and gag pol, we characterized the ability of the EIAV vector particles to form transducing units when pseudotyped with various envelopes (Figure 1d). pONY2.10lacZ and pONY3.0 were co-transfected with various envelope expression plasmids and the resulting vector particles were used to transduce three different cell lines, human kidney (293T), canine sarcoma (D17) and murine embryo (NIH3T3). For comparison an MLV vector (pHIT111)<sup>25</sup> and an HIV vector (pH4Z)<sup>5</sup> packaged into their respective gag/pol cores and pseudotyped with the various envelopes were included in the experiment. Data in Table 1 show that the EIAV vector system produced transducing particles with all of the envelopes used. Where tested, all three cell types were transduced except when the MLV ecotropic envelope was used, in which case, as might be expected, only the NIH3T3 cells were transduced. The titre varied between the different envelopes and cell types. This is the first report of efficient pseudotyping of a lentivirus with the rabies glycoprotein.

#### PCR analyses of transduced cells

In order to characterize the transduction events further we carried out a PCR analysis of 293T cells transduced by the EIAV vector (pONY2.10lacZ) pseudotyped with VSV-G. In particular, we asked if the vector genome, as opposed to a recombinant with the gag/pol expression plasmid, pONY3.0, had been the transduction vehicle for the lacZ gene. PCR amplification using primers specific for the EIAV LTR gave the expected PCR product of 310 bp when genomic DNA isolated from transduced cells was used (Figure 3a, lane 1). No PCR product was detected when mock-transduced 293T cell DNA was used as the template (Figure 3a, lane 2). pONY2.10lacZ was used as a positive control (Figure 3a, lane 3). No PCR product was detected when pONY3.0 was used as a template (Figure 3a, lane 4). The lack of a PCR product, when using pol-specific primers, (Figure 3b) confirmed that no gag/pol sequences from pONY3.0 had integrated into the host chromosomes. Taken together these data show that the authentic vector genome had transduced the cells.

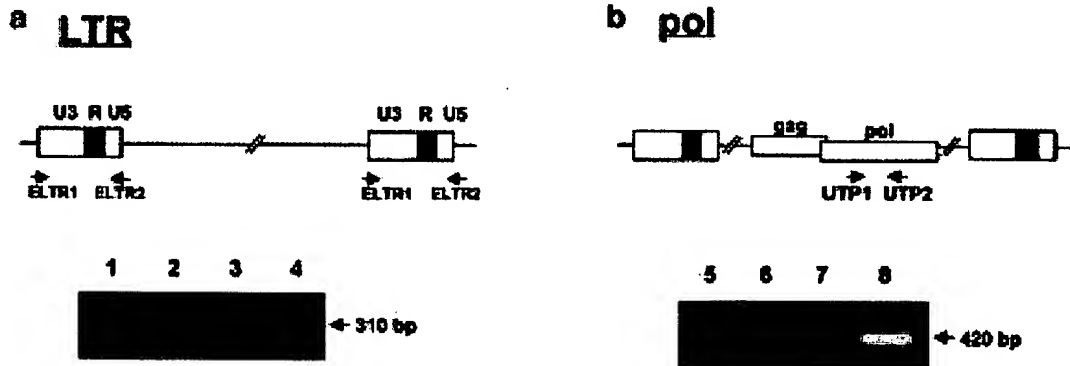
#### Minimal EIAV-based vector system

Having established that EIAV could be engineered into a vector system we sought to minimize the protein components that were expressed by the various cassettes. This should maximize the disablement of the virus and reduce any adverse effects in target cells that might be caused by expression of viral components or delivery of viral components by the particles. Initially, modifications to the vector genome were made. First, to minimize expression of gag/pol derivatives of pONY2.10lacZ were made. These contained nucleotides 1–3, 1–129, 1–206 or 1–373 bp of gag sequences. Using these constructs, the

**Table 1** Transduction efficiency of pseudotyped viral vectors

Vector	Envelope	Titre (i.f.u./ml) <sup>a</sup>		
		D17	NIH3T3	293T
EIAV	Mock	<1	<1	<1
EIAV	(MLVamp)	$1.0 \pm 0.6 \times 10^2$	$8.4 \pm 0.4 \times 10^2$	$2.0 \pm 0.9 \times 10^4$
EIAV	(MLVeco)	<1	$1.5 \pm 0.7 \times 10^4$	<1
EIAV	(VSVG)	$1.0 \pm 0.3 \times 10^5$	$3.6 \pm 0.5 \times 10^3$	$2.0 \pm 0.8 \times 10^5$
EIAV	(Rabies G)	$5.0 \pm 0.4 \times 10^4$	ND	ND
MLV	Mock	<1	<1	<1
MLV pHIT456	(MLVamp)	$1.3 \pm 0.4 \times 10^5$	$2.6 \pm 0.5 \times 10^6$	$2.0 \pm 0.2 \times 10^7$
MLV pHIT123	(MLVeco)	<1	$2.8 \pm 1.2 \times 10^6$	<1
MLV pRV67	(VSVG)	$3.0 \pm 0.5 \times 10^6$	$2.0 \pm 0.4 \times 10^5$	$5.0 \pm 0.7 \times 10^6$
MLV pRab	(Rabies G)	$8.0 \pm 0.3 \times 10^5$	ND	ND
HIV pRV67	(VSVG)	$4.0 \pm 0.1 \times 10^5$	<1	$5.0 \pm 0.3 \times 10^5$
HIV	Mock	<1	<1	<1

<sup>a</sup>Each cell type was transduced and stained for  $\beta$ -galactosidase activity 48 h after transduction of the target cells. Titres were averaged from three independent experiments and calculated as i.f.u. per ml. There was no more than 10% variation between experiments. Vector (pONY2.10lacZ) and envelope expression plasmids were co-transfected with the EIAV gag/pol expression plasmid (pONY3.0). pHIT111 and the envelope expression plasmids were co-transfected with the MLV gag/pol expression plasmid (pHIT60).<sup>25</sup> pH4Z and the VSV-G expression plasmid (pRV67)<sup>5</sup> were co-transfected with the HIV gag/pol expression plasmid (pGP-RRE3).<sup>5</sup> ND, not done.



**Figure 3** PCR analysis of EIAV vector-transduced 293T cells. PCR analysis of integrated EIAV vector. PCR was performed with either genomic DNA from EIAV vector-transduced cells (lanes 1 and 5) or mock-transduced cells (lanes 2 and 6). pONY2.10lacZ (lanes 3 and 7) and pONY3.0 (lanes 4 and 8) were used as controls. (a) PCR detection of EIAV LTRs. (b) PCR detection of pol.

optimal amount of gag sequence (based on titre) was found to be 373 bp. 1–3, 1–129, 1–206, 1–373 bp or 1–1376 of gag sequences gave titres of  $4.2 \times 10^2$ ,  $3.0 \times 10^5$ ,  $3.8 \times 10^5$ ,  $4.1 \times 10^5$  and  $1.0 \times 10^5$  lacZ-forming units (i.f.u.)/ml, respectively. pONY2.10lacZ contains 1376 bp of gag sequence. The construct containing this truncated gag gene was called pONY2.11 which should express a short gag peptide although this was not detectable by Western blot analysis (Figure 2). It should be possible to eliminate expression of the predicted remaining short peptide by mutation as has been achieved in retroviral vectors. To overcome dependence on tat for efficient production of vector genomes for packaging, the plasmid pONY4Z, was made (Figure 1c). In this construct the EIAV 5' U3 was replaced with the CMV promoter. We next sought to test the requirement for the two accessory functions, S2 and the dUTPase. A number of molecules were made which eliminated S2 from the system in both the vector genome plasmids and the gag/pol expression plasmids. Deletion of the start of S2 and env in the vector

genome plasmid pONY4Z produced pONY4ZΔS2. This construct contains the RREs and the tat and rev exons. A further construct pONY4.1Z removed these such that it contains only the 5' CMVLTR, leader, 373 bp of gag, the polypurine tract (PPT) and 3' LTR. The gag/pol expression plasmid pONY3.1 was modified to contain the same S2 and env deletion to produce pONY3.1ΔS2 (Figure 1b).

To test for the requirement for dUTPase the active site was mutated in pONY3.1 using the identical mutation identified by Steagall *et al*<sup>19</sup> that interferes with viral replication. The resulting plasmid was designated pONY3.1ΔU (Figure 1b). In addition, the sequences downstream of pol in pONY3.1 were replaced with a PCR fragment containing the putative RREs.<sup>14</sup> This was designated pHORSE3.1 (Figure 1b). This plasmid was found to be Rev dependent as has been observed for gag/pol expression in the HIV-based systems<sup>5,6</sup> (data not shown). pHORSE3.1 and pONY3.1 have the same leader sequence length. This is primer binding site to the start of gag and

is 140 bp long. The combination of pONY4.1Z vector genome with pHORSE3.1 provides a vector system that lacks tat, S2 and dUTPase.

#### Transduction of cell cycle arrested cells

Combinations of the plasmids described above were used to generate vector particles, pseudotyped with VSV-G, that were used to transduce dividing and non-dividing (aphidicolin arrested) D17 cells. The EIAV vectors particles were compared with the MLV and HIV systems (Table 2). These data firstly indicate the relative titres of the different vector genomes on dividing cells. We consistently observed that pONY2.11 gave about two- to four-fold higher titres than the less deleted pONY2.10-lacZ (data not shown). When U3 in the 5' LTR was replaced with the CMV enhancer/promoter as in pONY4 then titres increase a further five- to 10-fold. However, further deletion of the genome in pONY4.1Z gave a consistently lower titre than pONY4Z. This could be due to the absence of the RRE from the pONY4.1Z construct. In combination with pHORSE 3.1 titres were lowered further such that this combination gave on average five-fold lower titres than the combination of pONY4Z and pONY3.1. This reduction in titre may have been due to the configuration or sequence of the RREs or due to the employment of a four plasmid cotransfection system or because protein levels from pHORSE3.1 are slightly lower than from pONY3.1 (data not shown) and this is currently being addressed. As expected, none of the gag/pol expression plasmids produced transducing particles in the presence of a simple lacZ expression plasmid and none of the EIAV vector genomes produced any transducing activity in the absence of a gag/pol or env expression plasmid (unpublished data and Table 2). Integrases (IN) from most retroviruses, retrotransposons and

transposases share three highly conserved amino acids with a particular spacing (D<sub>51-58</sub>D<sub>35</sub>E) which if mutated leads to loss in function.<sup>1</sup> Mutation of the first of these amino acids in pONY3.1 (amino acid D64 to V64, nucleotide 4557 A to T) gave a drop in titre from  $4.0 \times 10^5$  to  $8.9 \times 10^2$  i.f.u./ml. When all three amino acids were mutated to D64 to Val64, D116 to A116 and E152 to A152 the titre dropped to  $1.3 \times 10^2$  i.f.u./ml. The colonies produced by transduction with the IN minus virus tended to be composed of single cells (over 95%) as opposed to the IN plus which tended to be composed of two or more cells (90%). This would indicate that the positive colonies in the IN minus transduction are episomal rather than integrated.

The increased transduction by EIAV of aphidicolin cells was consistently observed. We have no explanation for this although further experiments are being carried out to characterize this phenomenon.

Interestingly, mutation of S2 and dUTPase had no influence on the vector titres on dividing cells indicating that these proteins are not required for gene transfer and therefore suggesting that they are not necessary for vector particle integrity, yield, reverse transcription and gene expression. Over many experiments (data not shown) the titres of the most effective EIAV vector combinations were equivalent to those of a minimal HIV-based vector (pH4Z) system<sup>5</sup> or as shown in this study a few fold higher. When the transduction of the aphidicolin-arrested cells is analysed it can be seen that with all the lentiviral vectors the relative and the absolute titres are preserved. This indicates that the deletions in the genome and the mutations of the putative accessory factors, S2 and dUTPase, had no influence upon the ability of the vectors to transduce non-dividing cells *per se*. The pONY4Z vector gave consistently good titres of about  $5 \times 10^6$  i.f.u./ml on

Table 2 Transduction efficiency of viral vectors on dividing and non-dividing cells

Vector	gag/pol	Mean titre <sup>a</sup> (i.f.u./ml of viral stock)	
		Dividing cells	Growth arrested cells <sup>b</sup>
pONY2.11	pONY3.1	$4.1 \pm 0.2 \times 10^5$	$7.6 \pm 0.5 \times 10^5$
pONY4Z	pONY3.1	$4.0 \pm 0.5 \times 10^6$	$5.2 \pm 0.9 \times 10^6$
pONY4.1Z	pONY3.1	$1.0 \pm 0.7 \times 10^6$	$1.5 \pm 0.4 \times 10^6$
pONY4ZΔS2	pONY3.1	$5.0 \pm 0.4 \times 10^6$	$3.9 \pm 0.5 \times 10^6$
pONY4ZΔS2	pONY3.1ΔS2	$2.1 \pm 0.6 \times 10^6$	$4.4 \pm 1.2 \times 10^6$
pONY4ZΔS2	pONY3.1ΔU	$2.0 \pm 0.5 \times 10^6$	$1.3 \pm 0.8 \times 10^6$
pONY4Z	pONY3.1ΔS2	$4.7 \pm 0.8 \times 10^6$	$6.2 \pm 0.7 \times 10^6$
pONY4Z	pONY3.1ΔU	$2.8 \pm 0.6 \times 10^6$	$4.0 \pm 1.1 \times 10^6$
pONY4.1Z	pHORSE3.1 <sup>c</sup>	$8.1 \pm 0.4 \times 10^5$	$1.0 \pm 1.4 \times 10^6$
pONY4Z	pCI-Neo	<1	<1
pH4Z	pGP-RRE3	$8.1 \pm 0.5 \times 10^5$	$9.2 \pm 0.8 \times 10^5$
pH4Z	pCI-Neo	<1	<1
pHIT111	pHIT60	$4.0 \pm 1.0 \times 10^6$	$5.0 \pm 0.6 \times 10^5$
pHIT111	pCI-Neo	<1	<1

<sup>a</sup>Each cell type was transduced and stained for β-galactosidase activity 48 h after transduction of the target cells. Titres were averaged from three independent experiments and calculated as i.f.u. per ml. The EIAV vectors and the VSV-G envelope expression plasmid pRV67 were co-transfected with EIAV gag/pol expression plasmids. pHIT111 and the envelope expression plasmids were co-transfected with the MLV gag/pol expression plasmid (pHIT60).<sup>25</sup> pH4Z and the VSV-G expression plasmid (pRV67) were co-transfected with the HIV gag/pol expression plasmid (pGP-RRE3).<sup>5</sup>

<sup>b</sup>D17 cells were treated with aphidicolin (15 µg/ml) for 24 h before transduction and the medium was changed with fresh aphidicolin every 24 h.

<sup>c</sup>pCI-Neo Rev (4 µg) was also used in this transfection to provide Rev.

both dividing and non-dividing cells. This result was in marked contrast to the data obtained with the MLV vector system where titres on non-dividing cells were reduced by three orders of magnitude. Taken together, these data show that a high efficiency EIAV vector lacking all accessory and regulatory genes other than rev can be used for the transduction of both dividing and non-dividing cells.

#### Transduction of primary rat neuronal cultures

Having established that the EIAV-based vectors could effectively transduce growth arrested cells we next sought to establish whether they could be used to deliver genes to non-dividing primary cells *in vitro* using neurons as the target cells. Cultured dissociated hippocampal neurons from E18 rat embryos were allowed to differentiate in culture for a week and were then transduced using the pONY4Z and pONY3.1 system pseudotyped with VSV-G. Both *lacZ* (pONY4Z) and *GFP* (pONY4G) genes were used as markers for gene transfer. In addition, the S2 and dUTPase mutants were used. These EIAV vectors were again compared with HIV (pH4Z or pH3GFP) and MLV (pHIT111 or MLVGFP) vectors. Viral vector particles used to transduce these cultures was concentrated approximately 100-fold<sup>27</sup> (Figure 4). It was found that at an MOI of 10, 1, 0.1 and 0.01 (based on D17 transduction) the EIAV vectors expressing *lacZ* (Figure 4a) transduced differentiated neurons in these cultures about 100-fold more efficiently than the MLV vector (Figure 4b, showing data obtained at an MOI of 10). EIAV vectors made with the S2 deficient components (Figure 4d) and the dUTPase deficient components (data not shown) gave similar transduction efficiencies to the S2 and dUTPase vector (Figure 4c). EIAV vectors expressing *GFP* (pONY4G) also transduced hippocampal neurons efficiently (Figure 4e and f) with axons and dendrites clearly visible. A low but detectable level of transduction with the MLVGFP viral vector was observed (Figure 4h) but this was markedly less effective than either the pH3G (Figure 4g) and pONY4G (Figure 4e and f) viral vectors. This transduction by MLV observed with both reporters may be due to the presence of rare neural precursor cells known to exist in these preparations that were dividing during the transduction event. These data show that the accessory factors are not required for gene transfer to terminally differentiated cells indicating that they can be omitted from vectors.

To prove that the pONY4G-transduced cells in these cultures were post-mitotic, confocal microscopic analysis of immunofluorescence staining was carried out with an antibody against NeuN, a neuron-specific post-mitotic marker<sup>28</sup> and the fluorescent *GFP* (Figure 5). While the majority of untransduced neurons expressed NeuN, colocalization of both *GFP* and anti-NeuN in the nuclear and perinuclear compartments of neurons transduced with pONY4G viral vector was apparent (Figure 5c).

#### Transduction of adult rat brain *in vivo*

Having established that the EIAV-based vectors could transduce neuronal cells *in vitro* we next sought to establish the ability of EIAV viral vectors to transduce cells *in vivo*.

Stereotactic injections of pONY2.10*lacZ* viral vector into caudate putamen (corpus striatum) of adult rats ( $n = 7$ ) produced intense *LacZ* staining (Figure 6a and b)

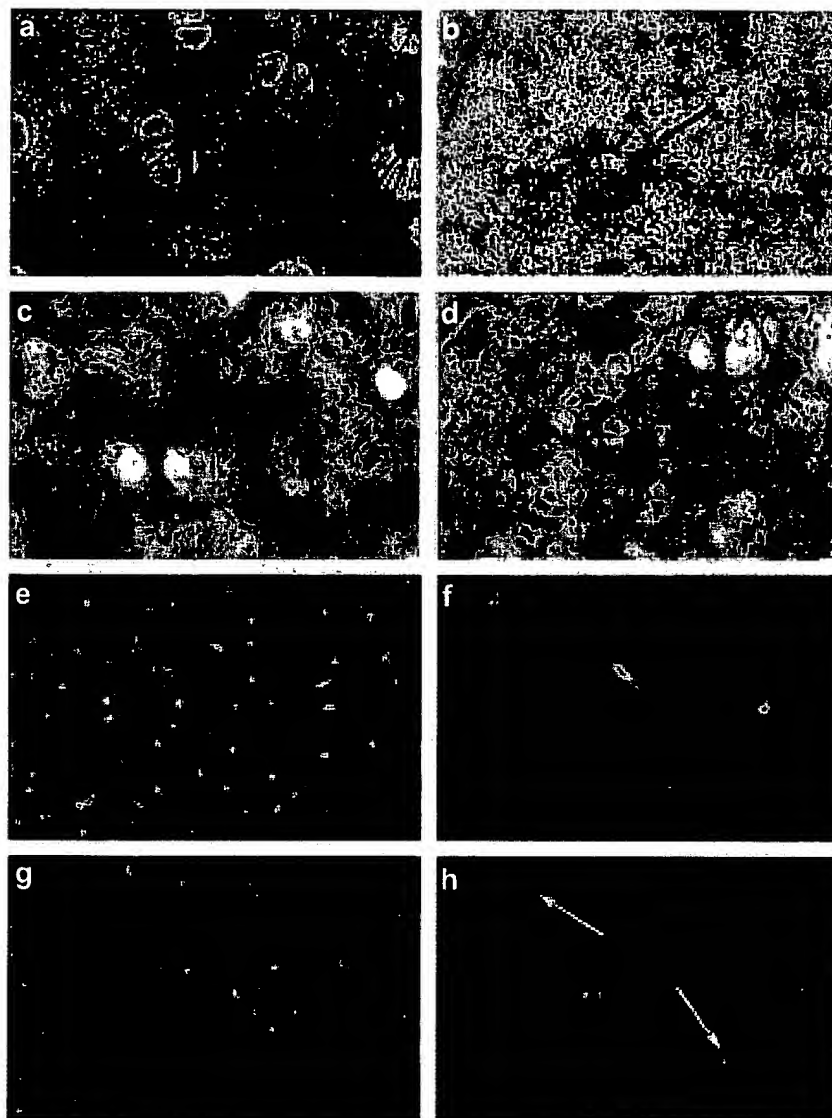
up to 28 days after injection (longest time-point examined). No *lacZ* expression was detected when mock virus (viral vectors prepared in the absence of the gag/pol plasmid, pONY3.1) was injected into the same site. Average transduction efficiency was  $1 \times 10^4$  cells, spanning the striatum. Analysis with the light microscope at 7 days after injection, showed no pathological change in the injected brain area despite a significant degree of viral vector clustering. Similar results were obtained following identical viral vector delivery into the dentate gyrus of the hippocampus in adult rats ( $n = 5$ ) (data not shown). Morphological examination of the cells transduced by EIAV demonstrated that in contrast to MLV-based vectors, EIAV transduced cells of both glial and neuronal appearance (unpublished data and Figure 6c and d). In addition to transduction of cells in caudate putamen, reporter expression was also observed in distinct neuronal nuclei of globus pallidus (Figure 6e, f and g), confirming the ability of this vector to transduce neurons in adult rat CNS. *In vivo* experiments with the EIAV minimal system are underway.

#### Discussion

Equine infectious anemia virus is the simplest lentivirus containing only three accessory proteins plus the dUTPase function encoded by *pol*. It has limited pathogenesis and replication is highly restricted to horse cells, in particular macrophages. We have been able to deconstruct the virus to produce a gene transfer vector that lacks all of the accessory functions except rev and remarkably this vector system functions in human cells and in rat neurons *in vitro* indicating that tissue tropism is broadened in the vector system. We were also able to demonstrate for the first time the transduction of differentiated post-mitotic mammalian neural cells both *in vitro* and *in vivo* with a non-primate lentivirus.

The lack of requirement for EIAV viral proteins other than gag and pol for gene transfer to non-dividing cells was somewhat surprising given that it is these proteins that distinguish the lentiviruses from the oncoretroviruses. It has been tempting to speculate that the propensity for lentiviruses to replicate in differentiated and non-dividing cells is enhanced by these proteins if not dependent upon them. However, recently the importance of sequences in gag and pol have also been emphasised.<sup>29–34</sup> Our findings are also consistent with the observations with HIV vectors that have been produced without accessory proteins apart from rev.<sup>5,6</sup> However, we cannot yet exclude the possibility that gene transfer might be enhanced by accessory factors in some cell types. Certainly, studies with HIV replication and HIV vectors have implicated a role for vpr in optimising gene transfer.<sup>6,35</sup> Our finding that S2 was not required is consistent with studies by Li *et al*<sup>36</sup> who have recently shown that EIAV S2 is not required for viral replication *in vitro* in a number of equine cell lines including monocyte-derived macrophages. In contrast, there have been studies that have suggested a requirement for the dUTPase for EIAV replication in macrophages but not dividing cells and this has been interpreted to mean that it may be required for infection of any non-dividing cells.<sup>17</sup> All of the non-primate lentiviruses encode a dUTPase within their *pol* genes. These enzymes act to lower the dUTP to dTTP ratio in cells during DNA replication, minimizing



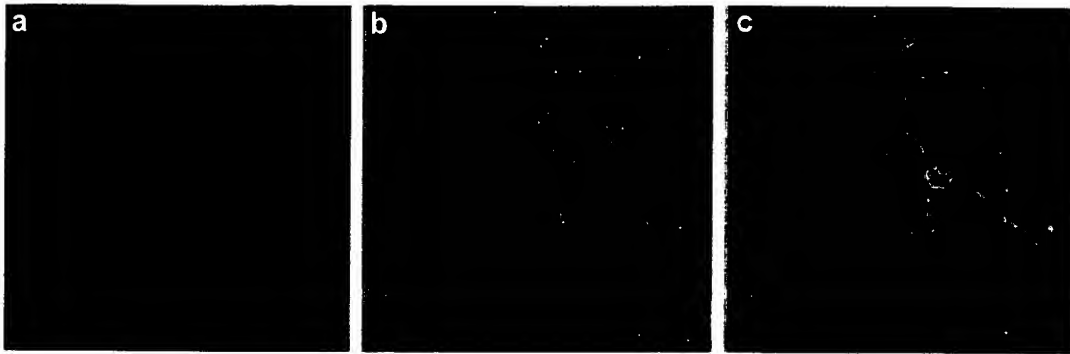


**Figure 4** *In vitro* transduction of cultured hippocampal neurons. Rat hippocampal neurons were transduced with various concentrated vectors all pseudotyped with VSV-G and used at an MOI of 5 to 10. (a, c) pONY4Z viral vector (plus pONY3.1); (b) pHT111 (plus pHT60); (d) pONY4ZΔS2 (plus pONY3.1ΔS2); (e, f) pONY4G (plus pONY3.1); (g) pHT3G (plus pGP-RRE3); (h) MLV-GFP (plus pHT60). (a, b, c, d, e, g, h) are at 20× magnification and (f) is at 60× magnification. (a and b) are phase contrast images, (c and d) are bright field and (e, f, g and h) are fluorescent images. Three independent preparations of viral vectors were made and used for transduction of three separate hippocampal neuronal preparations giving similar results in all cases. Arrows indicate some rare MLV transduced cells.

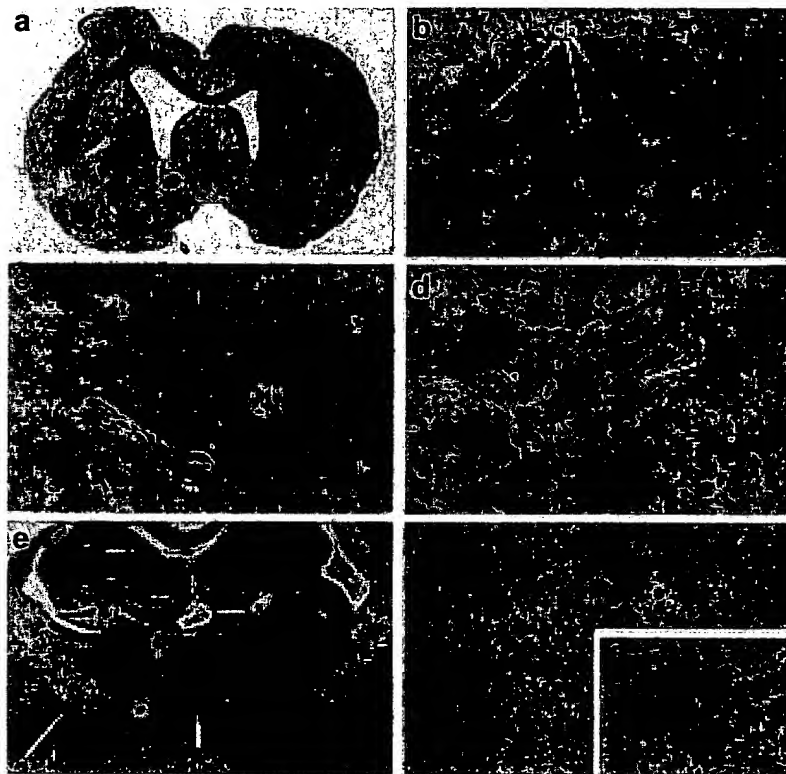
the incorporation of dUTP into DNA and hence lowering the mutation rate.<sup>22,23</sup> Cellular dUTPases are cell cycle controlled and either nuclear or mitochondrial<sup>37</sup> and are absent in quiescent cells. At G<sub>0</sub> cells have a high dUTP to dTTP ratio. In the absence of dUTPase dUTP is incorporated into the genome of viruses during reverse transcription leading to accumulation of G-to-A mutations<sup>22,38</sup> that would eventually inactivate the virus or lead to uracil glycosylase-mediated excision repair and DNA fragmentation. If the dUTPase is mutated in CAEV or EIAV it leads to a delayed replication in non-dividing primary macrophages.<sup>17–22</sup> Our data now show that this dUTPase activity is not required under conditions that essentially mimic a single cycle of infection. This is consistent with the proposed function of dUTPase that it is essentially

required to reduce a cumulative inhibition of viral replication induced by mutation load or to prevent excision repair. Removing the dUTPase activity from EIAV vectors could be considered a safety feature to minimize the potency of any replication competent virus. It appears, however, that human endogenous retroviruses (HERVs) might encode functional dUTPases which could substitute and thus explain why the primate lentiviruses do not encode a dUTPase.<sup>37</sup> We can therefore provide no evidence to support the notion that accessory functions in EIAV are absolutely required for gene transfer to non-dividing cells and we show that these functions can be deleted from gene transfer vectors without loss of titre and without reducing gene transfer efficiency on non-dividing cell lines and terminally differentiated neurons.





**Figure 5** Confocal immunofluorescence analysis of cultured hippocampal neurons. Rat hippocampal neurons were transduced with pONY4G viral vector (plus pONY3.1) pseudotyped with VSV-G and used at an MOI of 10. These were then stained with the Neu-N post-mitotic neuronal-specific marker (a), Anti-NeuN Texas red staining (b), GFP fluorescence (c), staining for NeuN and GFP fluorescence. Colocalisation of both fluorochromes produces yellow nuclear and perinuclear staining. (a, b and c) are at 60 $\times$  magnification.



**Figure 6** In vivo transduction of rat CNS. Rat brains were transduced with concentrated pONY2.10lacZ viral vector (plus pONY3.1) pseudotyped with VSV-G.  $2 \times 1 \mu\text{l}$  of viral vector were delivered into caudate putamen. The rats were killed from 2 days (e, f, g) to 28 days after transduction (a, b, c, d). (a) 50  $\mu\text{m}$  coronal section of rat striatum showing strong staining in caudate (indicated by arrow). Staining was only observed on the operated side and never on the contralateral (magnification 1.25 $\times$ ). (b) Higher magnification (30 $\times$ ) of transduced caudate in parallel coronal sections of the same brain, showing cells of both glial and neuronal morphology. Absence of staining of fibre bundles (marked fb) further confirm the striatal cell specificity of the LacZ staining. (c, d) Higher magnification (40 $\times$ ) of cell types transduced within caudate (c) neuron (d) neuron indicated by arrow in close association with glial cells. (e, f, g) show increasing magnifications (1.8 $\times$ , 15 $\times$  and 60 $\times$ ) of LacZ-stained neuronal nuclei of the medial globus pallidus, caudal to the site of injection. They consist of groups of fusiform neurons with large perikarya contacted by plexuses of afferent fibers<sup>50</sup> (indicated by \*, f and g). No staining was observed in the globus pallidus on the contralateral (unoperated) side. Examination of serial sections through the striatum of this rat showed that the transduction spanned part of the lateral globus pallidus and as such this staining could be either due to the transduction of striatofugal axons arborizing both pallidal segments or retrograde transport of pallidal neurons projecting to striatum.

We have provided evidence that it may be possible to pseudotype lentiviral vectors with a range of different envelopes albeit with variable efficiencies. The use of the rabies envelope may be particularly useful for accessing particular subsets of neurons and cells in the neuro-

muscular junction. The finding that the amphotropic envelope (4070A) can pseudotype EIAV opens the way to exploiting the wealth of information on envelope structure and targeting that has been obtained with this system.<sup>39,40</sup>

We have also attempted to reduce the vector genome to minimize the potential for any recombination during vector production and in target cells. The pONY4Z EIAV vectors lack 5.3 kb of the 8.2 kb viral genome and are capable of producing high-titre vectors. However, a more extensive deletion of 7 kb as in pONY4.1Z reduced efficiency. pONY4.1Z in combination with pHORSE3.1 provides a tat, S2 and env minus EIAV-based system although at a reduced titre compared with pONY4Z and pONY3.1. This was possibly due to the absence of the RREs although other effects on replication and gene expression cannot be excluded. It may be possible to render the EIAV system rev/RRE independent by either using a constitutive transport element (CTE) from Mazon-Pfizer monkey virus MPMV<sup>41,42</sup> or Simian retrovirus 1 (SRV-1).<sup>43</sup> Experience with MLV-based vectors indicates that further deletion and optimization of the vector configuration will be possible to give higher starting titres.<sup>25</sup> Such refinement will be important to facilitate the economic scale up of these vectors for clinical applications.

In conclusion, the simplicity of the EIAV genetic organisation has allowed us to construct a gene transfer vector that is highly potent for the transduction of non-dividing cells and in particular post-mitotic neurons. The vector production system only expresses the gag/pol proteins and rev so reducing any pathogenic potential, which in other lentiviruses largely resides with the envelope and accessory proteins.<sup>44–48</sup> The naturally restricted pathogenicity of EIAV and the low level of endemic infection further suggest that this vector system may have a high acceptability for use in many human gene therapy applications.

## Materials and methods

### Plasmid construction

Numbering used is as of Payne *et al.*<sup>24</sup> pONY 2.0 was created by linking a PCR amplified fragment comprising the EIAV LTR with the *MluI/MluI* (216/8124) fragment of a derivative of pSPEIAV19 that contained a 0.7 kb *HindIII/HindIII* deletion (5835/6571) in pBluescript II KS+ (Stratagene, La Jolla, CA, USA) (Figure 1a). In addition, part of the gag/pol region *BglII/NcoI* (1901/4949) was deleted and a  $\beta$ -galactosidase gene driven by the human cytomegalovirus immediate-early promoter/enhancer (CMV) was inserted. This was designated pONY2.10lacZ. pONY2.11 contains a larger deletion in gag such that only 373 bp of the gag ORF remains. pONY4Z was made by replacing the 5' U3 with CMV from pCI-Neo. pONY4.1Z contains a deletion downstream of the lacZ gene such that tat, S2, env, rev and RRE, are either missing or severely truncated (Figure 1c).

pONY4ZΔS2 contains a deletion from nucleotide positions 5345 to 5397 nt. This removes the ATG start codon of S2 and the start codon of env. The deletion was made by PCR using overlapping primers. pONY4G was made by replacing the lacZ gene of pONY4Z (*SacII/KpnI* and then blunting with Klenow polymerase) with that of GFP from pEGFP-N1 (*BamHI/XbaI*) and then blunting with Klenow polymerase as a blunt fragment (Clontech, Palo Alto, CA, USA). pH3G was made by inserting the GFP gene from pEGFP-N1 (Clontech) into pH3Z as an

*XhoI/SphI* fragment. MLVGFP (pHIT111G) was made by inserting the GFP gene from pEGFP-N1 (Clontech) into pHIT111 as a *SacII/NotI* fragment (a kind gift from M Yap, Biochemistry Department, Oxford University).

The EIAV gag/pol expression plasmid (pONY3.0) was made by inserting the *MluI/MluI* fragment from pONY2.0 into the mammalian expression plasmid pCI-neo (Promega) (Figure 1b). pONY3.1 is a derivative of pONY3.0 but has an additional 0.3 kb deletion at the 5' end (up to the *NarI* site just downstream of the primer binding site), removing all of the 5' LTR.

pHORSE3.1 was made by replacing all the EIAV sequence downstream of the pol stop codon with the EIAV RREs. The EIAV RREs were made by PCR using the sequence information from Martarano *et al.*<sup>14</sup>

pONY3.1ΔS2 contains a deletion from nucleotide positions 5345 to 5397 nt. This removes the ATG start codon of S2 and the start codon of env. The deletion was made by PCR using overlapping primers.

pONY3.1ΔU was made by site directed mutagenesis of nucleotide 4176 from a T to an A residue.<sup>19</sup> This mutates the aspartic acid to a glutamic acid.

pCI-NeoRev was made by PCR amplification of the EIAV REV exons (5507/5597 and 7295/7701 nt) using pSPEIAV19 as template.

### PCR analyses of transduced cells

Genomic DNA was isolated from transduced cells after passage for 14 days. PCR was carried out with primers specific for the LTR (region 61 to 382) and for the dUT-Pase (region 3956 to 4366).

### Production and assay of vectors

Vector stocks were generated by calcium-phosphate transfection of human kidney 293T cells plated onto 10 cm dishes with 16  $\mu$ g of vector plasmid, 16  $\mu$ g of gag/pol plasmid and 8  $\mu$ g of envelope plasmid. Thirty-six to 48 h after transfection, supernatants were filtered (0.45  $\mu$ m), divided into aliquots and stored at  $-70^{\circ}\text{C}$ . Concentrated vector preparations were made by ultracentrifugation at 50000 g (SW40Ti rotor) for 90 min, at  $4^{\circ}\text{C}$ . The virus was resuspended in PBS for 3–4 h, divided into aliquots and stored at  $-70^{\circ}\text{C}$ . Transduction was carried out in the presence of polybrene (8  $\mu$ g/ml).

### In vitro delivery of vectors to rat primary neurons

E18 timed-mated pregnant Wistar rats were obtained from Harlan (Bicester, UK), sedated with isoflurane and killed by cervical dislocation. Uterine horns were collected in Dulbecco's phosphate-buffered saline (Sigma, St Louis, MO, USA) and embryos transferred to Hank's balanced salt solution (HBSS; Sigma). Dissected hippocampi were incubated with 0.1% trypsin/0.05% DNase (Sigma) for 20 min at  $37^{\circ}\text{C}$  washed with 0.1% soyabean trypsin inhibitor (Sigma) in HBSS/DNase at  $37^{\circ}\text{C}$  and rinsed three times with HBSS/DNase. Complete dissociation of the cells was by trituration through a fire-polished pipette. Cells were counted and seeded at  $1.0 \times 10^5 \text{ cm}^{-2}$  in serum-free neurobasal media supplemented with B27 and L-glutamine (0.5 mM) (Gibco BRL, Grand Island, NY, USA). The cells were plated on to eight micro-well slides (Nunc, Coring, High Wycombe, USA) coated with 50  $\mu$ g/ml poly-D-lysine (Sigma), followed by 10  $\mu$ g/ml fibronectin adhesion peptide fragment (Sigma). Antibody staining for neuronal and glial markers established that these cultures are over 95% neuronal.

Primary cultures were transduced 4–7 days later by replacing culture media with media containing virus. Transduction was for 5 h at 37°C, after which half of the media were replaced with fresh media. Expression was investigated 48 h later either by X-gal staining or direct microscopy (GFP). For LacZ visualization, cultures were rinsed with PBS, fixed with 4% paraformaldehyde for 15 min, rinsed with PBS and reacted with X-gal at 37°C overnight and visualised by light microscopy.

For immunohistochemistry cultures were rinsed with PBS, fixed with 4% paraformaldehyde for 15 min and again rinsed with PBS. Labelling was performed by incubating sections for 24 h in Tris-buffered saline (TBS) with 10% goat serum and 0.3% Triton X-100, containing mouse monoclonal anti-NeuN (1:500, Chemicon, Temecula, CA, USA) antibody. Samples were then washed, blocked in TBS with 10% goat serum and 0.3% Triton X-100 containing goat anti-mouse antibody coupled to Texas Red (1:200; Molecular Probes, Eugene, OR, USA). Sections were mounted with Vectashield mounting medium (Vector, Burlingame, CA, USA) and analysed by confocal laser microscopy (Leica TCS NT, Heidelberg, Germany). Fluorescent images were collected, superimposed and processed for publication using Adobe Photoshop (Adobe System).

#### *In vivo delivery of vectors to adult rat brain*

Adult Albino Oxford (AO) rats were obtained from the colony at the Department of Human Anatomy, Oxford University. For intracerebral injections rats were anaesthetised with hypnorm and hypnovel,<sup>49</sup> placed in a stereotactic injection apparatus and two injections were delivered to the right caudate putamen using the coordinates: bregma, 3.5 mm lateral, 4.75 mm vertical from dura and 1 mm rostral, 3.5 mm lateral, 4.75 mm vertical. 1 µl of virus with a titer between  $3 \times 10^6$  and  $7.4 \times 10^7$  i.f.u./ml was used per injection, using a fine drawn glass micropipette over a period of 2 min and the pipette was left in place for an additional 2 min before withdrawal. Animals were killed 2, 7 or 28 days post-operatively using isoflurane and an intraperitoneal injection of euthanal, and perfused intracardially using 0.9% heparinised saline and either 4% cold paraformaldehyde or Zamboni's fixative (1.8% paraformaldehyde and 7.5% saturated picric acid). Brains were removed and placed in fixative overnight, saturated in 30% sucrose for a day and finally frozen in Tissue-Tek OCT embedding compound (Miles, IN, USA).

Coronal cryostat sections (50 µm) were collected in PBS and were stained with X-gal (37°C for 3 h to overnight) and visualized by light microscopy. X-gal-stained sections were counter-stained with Carbol Fuchsin, dehydrated and mounted.

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